Biology of Reproduction, 2017, 96(3), 686–693
doi:10.1095/biolreprod.116.144253
Research Article
Advance Access Publication Date: 2 February 2017

Research Article

Uniparental chicken offsprings derived from oogenesis of chicken primordial germ cells (ZZ)†

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[†]**Grant Support.** This research was funded by HH Sheikh Mohammed bin Rashid Al Maktoum, the Vice President and Prime Minister of UAE and Ruler of Dubai.

Received 17 August 2016; Revised 9 December 2016; Accepted 26 December 2016

Abstract

Cloning (somatic cell nuclear transfer) in avian species has proven unachievable due to the physical structure of the avian oocyte. Here, the sexual differentiation of primordial germ cells with genetic sex ZZ (ZZ PGCs) was investigated in female germline chimeric chicken hosts with the aim to produce uniparental offspring. ZZ PGCs were expanded in culture and transplanted into the same and opposite sex chicken embryos which were partially sterilized using irradiation. All tested chimeric roosters (ZZ/ZZ) showed germline transmission with transmission rates of 3.2%–91.4%. Unexpectedly, functional oogenesis of chicken ZZ PGCs was found in three chimeric hens, resulting in a transmission rate of 2.3%–27.8%. Matings were conducted between the germline chimeras (ZZ/ZZ and ZZ/ZW) which derived from the same ZZ PGCs line. Paternal uniparental chicken offspring were obtained with a transmission rate up to 28.4% and as expected, all uniparental offspring were phenotypic male (ZZ). Genotype analysis of uniparental offsprings was performed using 13 microsatellite markers. The genotype profile showed that uniparental offspring were 100% genetically identical to the donor ZZ PGC line, shared 69.2%–88.5% identity with the donor bird. Homozygosity of the tested birds varied from 61.5% to 84.6%, which was higher than the donor bird (38.5%). These results demonstrate that male avian ZZ PGCs can differentiate into functional ova in an ovary, and uniparental avian clones are possible. This technology suggests novel approaches for generating genetically similar flocks of birds and for the conservation of avian genetic resources.

Summary Sentence

Cultured chicken primordial germ cells (ZZ) possess the developmental plasticity to differentiate into functional ova in the ovary of germline chimeric chicken host, and give rise to uniparental chicken offspring.

Key words: gametogenesis, sexual differentiation, primordial germ cells, germline chimera.

Introduction

In many species, sex determination and sexual differentiation into sex-related phenotypes are events of genetic programming. The genetic gender is determined at the moment of fertilization. In birds, the male possesses the homogametic sex (ZZ), and the heterogametic sex is the female (ZW) [1]. The early precursors of the gametes, the primordial germ cells (PGCs), are formed early in development and

migrate to colonize the forming gonad. Germ cell differentiation is guided by the embryonic milieu in many species. Under influences of sex hormones and the surrounding somatic cells, male germ cells in the testes differentiate into the resident spermatogonia and form spermatozoa, while in females the germ cells enter meiosis and differentiate into the ova. Primordial germ cells are the progenitor cells of germ cell lineage. Several key pluripotency genes (such as *Oct4*, *Sox2*, *Nanog*, etc.) are expressed in PGCs [2], and PGCs from various stages of early embryo showed plasticity in their developmental fate in vitro and in vivo. In many vertebrate species, the germ cells will follow the somatic environment and form functional gametes when transplanted into the opposite sex. In the XX testis of sex reversed mouse, XX germ cells enter the first phase of spermatogenesis and become prospermatogonia. In the XY ovary, XY germ cells enter meiosis and differentiate as primary oocytes, the fertility depends on the species, genetic background and causes of sex reversal [3]. In fish, ZZ germ cells will form functional ova when transplanted into female host embryos [4]. In birds, the situation is less certain. When blastoderm cells containing precursors of PGCs [5], migratory PGCs [6], and gonadal PGCs [7, 8] were transferred into germ cell-ablated, reverse sex hosts, offspring from the transplanted PGCs were reported at extremely low frequencies. In those studies, the number and purity of donor PGCs were limited. In a defined culture system, chicken PGCs could keep proliferating for a long period and reprogrammed into a germline competent cell line [9]. A high rate of germline transmission was obtained by transferring cultured PGCs. It was reported that cultured PGCs did not form functional gametes in sex reversed hosts [10]. In this study, Barred Plymouth Rock chicken ZZ PGCs were cultured and transplanted into both male and female irradiated White Leghorn chicken hosts. The oogenesis of chicken ZZ PGCs in the ovary of chimeric chicken was investigated. Donor-derived offspring were produced from both sexes. Furthermore, paternal uniparental chicken offspring were produced from the functional spermatozoa and ova of sole genetic origin through male and female germline chimeras. Uniparental chicken offspring may provide desired experimental materials for avian gametogenesis research, and also novel approaches for breeding and conservation of avian genetic resources. (The experimental design was illustrated in Figure 1.)

Materials and methods

Barred Plymouth Rock (homozygous recessive i/i) chickens, White Leghorn chicken (homozygous dominant I/I) layers and chimeric birds were kept in the chicken house at the Central Veterinary Research Laboratory (CVRL), Dubai, UAE. All experimental animals and treatment in this study were reviewed and approved by the Animal Ethic Committee of CVRL, and Ministry of Climate change and Environment of the UAE, according to the Ministerial Decree No. 384 of the year 2008 on the executive by-law of the Federal Law No. 16 of the year 2007 concerning animal welfare.

Culture and characterization of Barred Plymouth Rock chicken primordial germ cells

Barred Plymouth Rock chicken PGCs were cultured as described by van de Lavoir et al. [10]. Briefly, 2–3 μl blood samples were collected from 123 Barred Plymouth Rock chicken embryos (stage 14, H&H). The embryos were sealed with double layers of parafilm, and returned to incubator. Donor embryos from which optimal PGCs culture derived were allowed to hatch. Blood samples were seeded on mitotically inactivated mouse fibroblast feeders (STO, ATCC

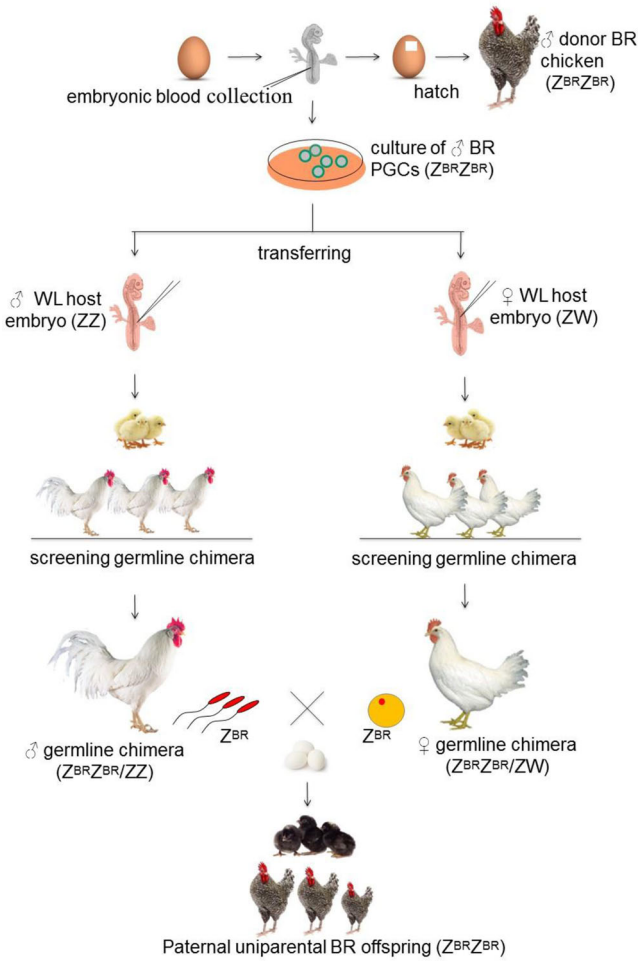

Figure 1. Diagram of experimental design and procedures.

CRL-1503, Manassas, USA) with Knockout Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 50% Buffalo Rat Liver cells (BRL 3A, ATCC CRL-1442, Manassas, USA) conditioned medium, 7.5% fetal bovine serum, 2.5% chicken serum, 1× nonessential amino acid, 1× nucleosides, 10 mM sodium pyruvate and β-mercaptoethanol. Fibroblast growth factor 2 and stem cell factor were added at a concentration of 10 ng/ml each. The cultures were maintained in 5% CO_2 incubator at 37°C. Cells were passaged at 3–4 days interval. A density of 5×10^4 cells were seeded in each well of 24-well plate (Falcon, Corning).

Primordial germ cells were fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, and incubated in blocking buffer (PBS/5%BSA/0.5%Tween-20) for 1 h. Anti-SSEA-1 (stage-specific embryonic antigen 1, MC480, 1:200) was added and incubated at 4°C overnight, then with secondary antibody biotinylated goat anti-mouse IgM (BA2020, Vector laboratories, Burlingame, USA) for 1 h. Texas Red Avidin D (A-1100, Vector laboratories, Burlingame, USA) was added at 1:500 for 1 h, washed with PBS (-), and mounted with Vectashield/DAPI. Slides were observed under fluorescent microscope. Chicken VASA homolog was also detected with the same procedure, except using rabbit anti-CVH (1:500, a gift from Dr. Noce, Keio University, Japan) and rabbit polyclonal anti-DDX4/MVH (mouse VASA homolog, Abcam ab13840) as primary antibody, and goat anti-rabbit biotinylated (1:200) as secondary antibody. Primordial germ cells omitting primary antibody incubation were used as negative control. The experiment was repeated twice.

To detect gene expression profiles of chicken PGCs, total RNA was extracted using the RNeasy mini kit (Qiagen). RT premix was prepared as per the manufacturer's instruction. Premix (15 μl) was added to 10 μl of total RNA (1 μg). Complementary DNA was obtained using Ipsogen RT kit (Qiagen). The Polymerase chain reaction (PCR) primers for detecting the expression of *cPouv, cNanog, cKlf4, cSox2, cMyc, Cvh, and Gapdh* genes were applied as published [10]. Primers detecting *cDazl* gene were designed: forward 5′AGAAGGAAAAATCATGCCAAA, reverse 5′ATTGCTGGTCCCAGTTTCAG. PCR was performed using Taq PCR master mix kits (Qiagen). Reaction mixture was prepared as 12.5 μl master mix, 1.25 μl forward primer, 1.25 μl reverse primer, 8 μl H2O, 2 μl cDNA. PCR was run at 95°C, 20 min; 95°C, 30 s; 55°C–59°C, 30 s; 72°C, 1 min; 60°C for 30 min. PCR products were loaded on 1% agarose gel.

Transplantation of Barred Plymouth Rock chicken ZZ primordial germ cells into White Leghorn chicken embryos

Freshly laid White Leghorn chicken eggs were collected, and irradiated with an X-ray facility at a dose of 600 rads in Al Tawam Hospital, Al Ain, UAE. The nonirradiated normal eggs were used as control. All the recipient eggs were incubated to stages 16–17 (H&H). To expose the embryo, a small piece of egg shell was removed from the sharp end of eggshell. Barred Plymouth Rock chicken ZZ PGCs in culture were harvested by gently pipetting, centrifuged, and resuspended to a concentration of 2×10^3 cells/μl in the culture medium. A number of 4,000 PGCs in 2 μl culture medium were injected into the dorsal aorta of recipient White Leghorn chicken embryos [11]. Two ZZ PGC lines (1110 g.64, 1110 h.79) were selected as donor cells to produce germline chimera. The injected eggs were sealed with double layer of parafilm, and incubated at 37.8°C with 60% relative humidity to hatch.

Progeny test of chimeric chickens

The chimeric birds were raised to sexual maturity under common condition. To evaluate the contribution of donor PGCs in the germline of recipient birds, 28 putative male chimeras and 27 female chimeras (ZZ/ZW) were selected for progeny test through artificial insemination. The resulting black chicks with Barred Plymouth Rock plumage were considered as donor PGCs-derived offspring.

Phase 1: screening male germline chimera (ZZ/ZZ) and female germline chimeric females (ZZ/ZW)

The semen samples were collected individually from 28 putative male chimeras (ZZ/ZZ), and inseminated into normal Barred Plymouth Rock females. Twenty-seven chimeric females (ZZ/ZW) were inseminated with normal Barred Plymouth Rock chicken semen. Insemination was conducted once a week for 3 months. The resulting eggs were collected and incubated to hatch.

Phase 2: generation of uniparental Barred Plymouth Rock chicken offspring

Two male germline chimeras (ZZ/ZZ) and three female chimeras (ZZ/ZW) showed high rate of germline transmission, and were crossbred through artificial insemination. The resulting eggs were incubated to hatch. The dead embryos during the last week of incubation were opened to check the feather phenotype. Molecular sexing was carried out by PCR with feather samples from the resulting chicks with Barred Plymouth Rock phenotype. These chicks were raised to sexual maturity under common condition, and bred with normal Barred Plymouth Rock females to confirm the fertility.

Molecular analysis of paternal uniparental chicken offspring

The genotype analysis of genomic DNA was carried out using 13 chicken microsatellite markers (Supplemental Table S3) [13–15]. The 5′ end of the forward primer was labeled with a universal M13 forward or M13 reverse tail. Fluorescent labeled M13 primers were used to detect amplified products. The primers for various loci were multiplexed in the way that the amplified product sizes did not overlap. Genomic DNA of uniparental offsprings, surrogate parents, the donor bird, and PGCs line was extracted using the Qiagen DNeasy blood and tissue extraction kit. Extracted DNA was checked using the Nanodrop (Thermo Scientific), and diluted to a concentration of 30–50 ng/μl. The PCR was performed in a total volume of 11 μl mixture containing 30–50 ng genomic DNA, 2 mM MgCl2, 200 μM of each dNTP, 1U GoTaq polymerase (Promega Inc., WI). The PCR cycling conditions were as follows: 95°C for 3 min followed by 35 cycles of 95°C for 15 s, 54°C for 30 s, 72°C for 30 s followed by final extension at 72°C for 45 min and 60°C for 30 min. Normal chicken genomic DNA was used as positive control, and nontemplate PCR mix as negative control. Amplified PCR products were mixed with Hi-Di formamide, internal size standard GeneScan-Liz 500 (Applied Biosystems), denatured at 95°C, and snap cooled. Size analyses of the separated DNA fragments were performed using the GeneMapper V4 software (Applied Biosystems).

Statistical analysis

The proportion data of germline transmission frequencies were arcsine transformation, then analyzed with the Student *t*-test. The hatchability differences were subjected to the χ^2 test. Statistical analysis was performed on SPSS software version 20 (Statistical Analysis System, SAS Institute, 1990). Statistical significance was accepted at $P < 0.05$.

Results

Derivation of Barred Plymouth Rock chicken primordial germ cell line from embryonic blood samples

A total of 123 blood samples (59 male, 64 female) were collected and cultured. Primordial germ cells divided slowly in the first a few days, and were seen after a week. Cells attached loosely on the STO feeder layer, and were harvested by gentle pipetting. After 20–30 days culture, a total number of five ZZ PGC cultures grew robustly (Figure 2a) with a population doubling time about 20 h, were maintained for transplantation, and frozen down. ZW PGCs showed different growth pattern, proliferated slower than ZZ PGCs, and aggregated into tightly compacted clumps floating on the feeder layer. In this study, two ZW PGC lines were obtained from 64 primary cultures. Out of the 59 male embryos, 15 chicks hatched with a hatchability of 12.2%. Two ZZ PGC lines (1110 g.64 and 1110 h.79) were selected and cultured for 38–49 days for chimera production.

Cultured chicken PGCs were stained positively with the antibody recognizing SSEA-1 and anti-CVH, weakly stained with anti-DDX4/MVH antibody. No signal was shown with the control cells when omitting primary antibody (Figure 2c). Pluripotency-associated genes (*cPOUV, cNANOG, cSOX2*), oncogenic genes (*cMYC and cKLF4*), and germ cell specifically expressed genes

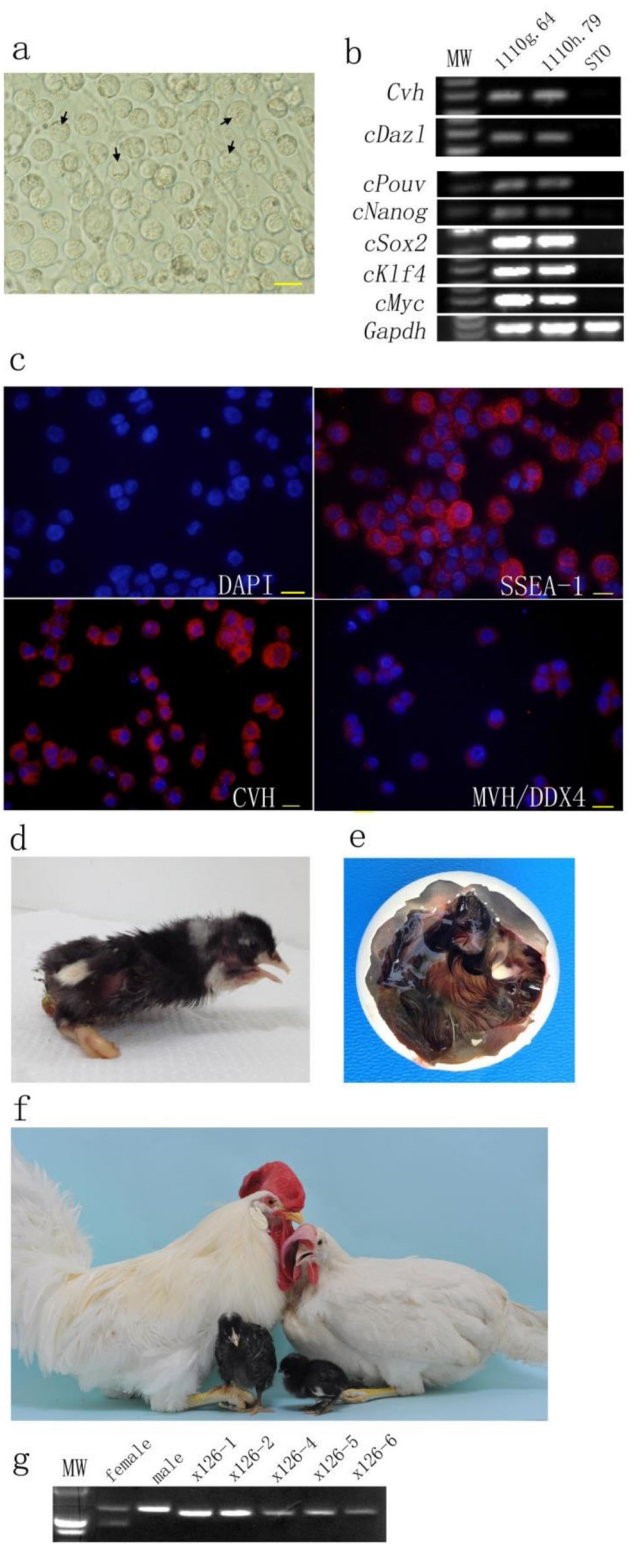

Figure 2. Culture, characterization of chicken PGCs, and generation of paternal uniparental offspring (scale = 20 μm). (a) Barred Plymouth Rock chicken PGCs (arrow, 1110 g.64) 38 days in culture. (b) Gene expression profile of cultured chicken PGCs (*Cvh, cDazl, cPouv, cNanog, cSox2, cKlf4, and cMyc*). (c) Characterization of chicken PGCs with anti-SSEA-1, anti-CVH, and anti-MVH. (d) Newly hatched paternal uniparental offspring. (e) A dead uniparental embryo. (f) Heterosexual (ZZ/ZW) chimeric hen (x126), germline chimeric rooster (x104), and their paternal uniparental offspring. (g) Molecular sexing of uniparental offspring.

(*DAZL, CVH*) were detected from the two ZZ PGC lines. PCR products were amplified from the cDNA samples with all eight set primers. These results in accordance with literatures of other researchers [9, 10] suggested that cultured PGCs expressed *POUV, NANOG, KLF4, SOX2*, and *c-MYC* homologous genes, and the expression of *DAZL* and *VASA* confirmed their germ cell lineage identity (Figure 2b).

Production of male (ZZ/ZZ) and female (ZZ/ZW) chicken germline chimeras

A total number of 214 White Leghorn chicken eggs were injected with chicken ZZ PGC lines (1110 g.64 and 1110 h.79), and 80 eggs hatched with the hatchability of 37.4%. Among the injected eggs, 34.5% (38/110) of the irradiated eggs hatched, and 40.4% (42/104) of the eggs hatched from the nonirradiated control eggs. There was no significant loss of hatchability with X-ray irradiation (χ^2 test, $P > 0.05$). A total number of 80 chimeric chicks were raised under normal condition. Seventy birds (38 male chimeras and 32 ZZ/ZW type female chimeras) survived at 2 months old (Table 1).

Germline transmission of male (ZZ/ZZ) chimeric chicken

Twenty-eight (100.0%) birds produced donor PGCs-derived black Barred Plymouth Rock chicks in the male chimeras (ZZ/ZZ), and were confirmed as germline chimera. Donor PGC line 1110 g.64 derived Barred Plymouth Rock chicken progenies was 54.1 ± 23.3% in nonirradiated recipient eggs and 81.7 ± 10.1% in irradiated recipients (Table 2). In the chimeric roosters (ZZ/ZZ) from PGC line 1110 h.79, the proportion of Barred Plymouth Rock chicken offspring was 53.3 ± 24.3% in nonirradiated recipient eggs, and 54.5 ± 12.5% in irradiated recipients (Supplemental Table S1). Prior to introducing donor PGCs, irradiation of recipient embryos significantly increased the germline transmission rate in chimeras with cell line 1110 g.64 (Student *t*-test, $P < 0.05$); however, there was no significant difference in the birds transplanted with PGC line 1110 h.79.

Germline transmission of opposite-sex chimeric females (ZZ/ZW)

Among 11 chimeric females (ZZ/ZW) from the PGCs line 1110 g.64, three birds (no. 17, ×103, x126) produced donor-derived Barred Plymouth Rock chicken offsprings with the transmission rate of 2.3%, 2.6%, and 27.8% (Table 3, Figure 2d–f), respectively. All of the black offsprings obtained from female chimeras (ZZ/ZW) were identified as males (ZZ) by molecular sexing with PCR (Figure 2g). However, the 16 chimeric females (ZZ/ZW) from the PGC line 1110 h.79 did not produce any Barred Plymouth Rock chicken offsprings (Supplemental Table S2).

Generation of uniparental Barred Plymouth Rock chicken offsprings

Three chimeric females (ZZ/ZW; bird no.17, x103, and x126) and two chimeric roosters(ZZ/ZZ; bird x104, x143), which showed high germline transmission rate in the progeny test phase I (Tables 3 and 4), were chosen to participate the testcrossing in phase 2 with the aim to produce uniparental offspring. The combinations of these birds were set as follows (Table 4).

A total number of 20 offsprings with Barred Plymouth Rock chicken phonotype were obtained from the two chimeric females

Table 1. Transplantation of cultured Barred Plymouth Rock chicken PGCs (ZZ) into White Leghorn chicken embryo.

			Chimera production			Survival at 2 months old		
Group	Recipient WL eggs	Donor BR PGCs (ZZ) line	No. eggs injected	No. eggs hatched	Hatchability (%)	Male ZZ/ZZ	Female ZZ/ZW	Total
1	Normal WL	1110 g.64	45	19	42.2	9	7	16
2	Irradiated	1110 g.64	55	20	36.4	11	7	18
3	Normal WL	1110 h.79	59	23	39.0	12	8	20
4	Irradiated	1110 h.79	55	18	32.7	6	10	16

Table 2. Progeny test of male chimeras (ZZ/ZZ) from PGC line 1110 g.64.

Group	Bird ID	No. of white offspring	No. of black offspring	Total No. of offspring	Rate of germline transmission (%)
Group 1 normal recipient	2	10	51	61	83.6
	3	46	16	62	25.8
	36	15	47	62	75.8
	39	9	5	14	35.7
	40	10	7	17	41.2
	44	32	53	85	62.4
	Means ± SD				54.1 ± 23.3
Group 2 irradiated recipient	x104	5	43	48	89.6
	x108	5	26	31	83.9
	x116	3	10	13	76.9
	x129	18	55	73	75.3
	x140	3	32	35	91.4
	x142	10	18	28	64.3
	x143	3	29	32	90.6
	Means ± SD				81.7 ± 10.1*

*Student *t*-test, $P < 0.05$.

Table 3. Progeny test of opposite-sex chimeric females (ZZ/ZW) with PGC line 1110 g.64.

Group	Bird ID	No. of white offspring	No. of black offspring	Total no. of offspring	Rate of germline transmission (%)
Group 1 normal recipient	5	38	0	38	0
	17	42	1	43	2.3
	21	48	0	48	0
	27	23	0	23	0
	42	38	0	38	0
	total	**189**	**1**	**190**	**0.5**
Group 2 irradiated recipient	x103	38	1	39	2.6
	x125	61	0	61	0
	x126	13	5	18	27.8
	x132	58	0	58	0
	x150	30	0	30	0
	x152	44	0	44	0
	Total	**244**	**6**	**250**	**2.4**

(ZZ/ZW). The bird x126 showed 28.4% (19/67) of germline transmission rate. These Barred Plymouth Rock offsprings were paternal uniparental offspring derived from the solo genetic source cell line 1110 g.64. Molecular sexing results showed that all of the 20 Barred Plymouth Rock offsprings were male (ZZ). A number of six offsprings died during the third week of incubation, and seven chicks died in the first week after hatch, seven survived to sexual maturity. The paternal uniparental offsprings showed high mortality which could be caused by high extent of homozygous genotype.

Molecular analysis of paternal uniparental Barred Plymouth Rock chicken offsprings

Thirteen individual microsatellite primer pairs were used (Table 5). Nine PCR products were found specific to genomic DNA of donor PGCs line, with the sizes of 190 bp (MCW14), 336 bp (MCW183), 153 bp (MCW81), 133 bp (HUJ12), 144 bp (HUJ2), 165 bp (MCW216), 120 bp (MCW295), 245 bp (MCW34), and 173 bp (MCW69). Particularly, three primer pairs (MCW14, MCW183, and MCW81) showed homozygous loci with the genomic DNA of donor PGCs line, and produced specific PCR product size of

Table 4. Testcross between male *(ZZ/ZZ)* and female (ZZ/ZW) chimeras from the same PGC line (1110 g.64).

Combination ♂-♀	No. of eggs	Fertility (%)	No. of offspring			Incubation and survival of black offspring			
			White offspring	Black offspring	% of black offspring	<13 *dpi**	14–21*dpi*	Died first week	Adult
x104-x126	83	81.9	48	19	28.4	N/D	6	7	6
x143-x103	74	32.4	23	1	4.2	N/D	0	0	1
x143-no.17	76	55.3	42	0	0	N/D	0	0	0

dpi: days post incubation.

Table 5. Genotype of paternal uniparental Barred Plymouth Rock offspring from chimeras (ZZ/ZZ and ZZ/ZW).

Marker	Genetic parent		Genotype of uniparental Barred Plymouth Rock offspring						Surrogate parents	
	Donor bird BPR64	Donor cell line 1110 g.64	x126-1	x126-2	x126-3	x126-4	x126-5	x126-6	Chimera (ZZ/ZZ) x104	Chimera (ZZ/ZW) x126
MCW111	117/117	117/117	117/117	117/117	117/117	117/117	117/117	117/117	117/117	117/115
MCW14	**190/190**	190/190	190/190	190/190	190/190	190/190	190/190	190/190	204/206	204/206
MCW183	**336/336**	336/336	336/336	336/336	336/336	336/336	336/336	336/336	312/312	324/320
MCW284	263/263	263/263	263/263	263/263	263/263	263/263	263/263	263/263	263/263	263/263
MCW81	**153/153**	153/153	153/153	153/153	153/153	153/153	153/153	153/153	131/131	131/131
HUJ12	**133**/153	133/153	133/153	153/153	133/133	133/153	153/153	133/153	137/153	135/153
HUJ2	**144**/158	144/158	144/144	144/158	158/158	144/144	144/144	144/158	148/158	148/150
MCW216	**165**/167	165/167	165/167	165/165	165/167	167/167	167/167	165/167	167/167	167/167
MCW295	106/**120**	106/120	106/120	106/120	106/120	106/106	106/120	120/120	104/102	106/104
MCW330	284/292	284/292	284/284	284/292	292/292	284/284	ND	284/292	284/292	292/292
MCW34	241/**245**	241/245	241/245	245/245	241/245	245/245	ND	241/241	241/249	251/253
MCW67	195/199	195/199	195/195	195/199	199/199	195/199	195/ND	195/195	195/199	195/195
MCW69	**173**/179	173/179	173/173	173/173	173/179	173/173	173/179	173/179	179/179	179/179
Homozygosity	5/13	5/13	9/13	9/13	9/13	11/13	8/13	8/13	6/13	6/13
Identity	26/26	26/26	22/26	22/26	22/26	20/26	18/26	23/26		

Note:

1. The numbers stand for the PCR product fragment size by base pair. *ND: not detected duo to technical failure.

2. X126-1,2,3,4,5,6: the six paternal uniparental Barred Plymouth Rock chicken offspring from chimeric rooster (x104, ZZ/ZZ) and chimeric female (x126, ZZ/ZW).

3. In donor PGCs line (1110 g.64), five loci markers (MCW111, MCW14, MCW183, MCW284, and MCW81) show homozygous. The remaining eight loci are heterozygous.

4. The numbers in bold show the eight loci which gave specific size of PCR product in the genomic DNA of donor bird only, and not existed in the chimeras. The shaded numbers are the heterozygous loci which uniparental offspring inherited from the uniparent.

190 bp, 336 bp, and 153 bp, respectively. With the 13 primer pairs, genotype of the PGC line (1110 g.64) was 100.0% identical to the donor Barred Plymouth Rock rooster (BPR64). Uniparental offsprings showed only donor specific PCR products in the three homozygous loci with primer pairs (MCW14, MCW183, and MCW81). In addition, all of the uniparental offsprings showed the Barred Plymouth Rock (homozygous recessive i/i) plumage phenotype. These genotyping results confirmed that uniparental offsprings genetically originated from the donor PGCs line (1110 g.64) only, and were nonrelated to the surrogate parents (White Leghorn chimeric chicken x104 and x126) (Supplemental Figure S1). Among the 26 alleles of 13 loci, 18 to 23 alleles of uniparetal offsprings were identical to the donor bird (BPR64), indicating that the genome of uniparental offsprings were 69.2% (18/26) to 88.5% (23/26) identical to that of donor bird (BPR64). Among the 13 loci of genomic DNA in donor bird, 5 (38.5%) loci (MCW111, MCW14, MCW183, MCW284, and MCW81) showed homozygous. However, in the six uniparental offsprings tested, 8 to 11 loci were found homozygous, indicating the homozygosity of 61.5% (8/13) to 84.6% (11/13), which was much higher than that of the donor bird (38.5%). These results suggested that uniparental offsprings bred through "self-mating" inherited higher genetic content of the only uniparent, and greatly improved the homozygosity than conventional breeding.

Discussion

Primordial germ cells are the progenitor cells of germline development. In birds, precursors of PGCs were first identified as about 30 cells expressing *Vasa* homolog gene (*Cvh*) in the central zone of area pellucida at stage X [15]. In mammal, PGCs proliferated in vitro and reprogrammed into embryonic germ cells in a medium with the addition of several growth factors [16, 17]. However, in similar culture conditions, chicken PGCs proliferate for long term, and are still capable to repopulate in the gonad of recipient birds, giving rise to donor-derived offspring [9, 10]. In this study, a number of five (8.5%) ZZ PGC lines were established from 59 cultures of male samples, and two (3.1%) ZW PGC lines were obtained from 64 cultures of female samples. In general, ZZ PGCs proliferate faster than ZW PGCs in primary cultures, but also individual variation was found among the cultures of both sexes [18]. In most cases, ZW PGCs divide a couple of times, form tightly compacted clumps in the early passages, and proliferate slowly. Chicken PGCs were found expressing pluripotency genes (such as *Oct-4, Sox2, Nanog,* etc.) at early

stage. The transcription of these genes was gradually downregulated from stage 14 to 28 [2]. As a consequence, more PGCs might progressively lose the pluripotency in advanced developmental stages. The proliferation discrepancy of PGCs in culture may attribute to their developmental heterogeneity. In similar studies, Song et al. reported that the culture of PGCs has a higher rate of success from germinal crescent area than those from blood source and primitive gonadal tissues [19]. Long-term cultured PGCs were reported to keep expressing pluripotency genes and germline-specific genes. Our present study also confirmed the expression of c*Pouv, cNanog, cKlf4, cSox2, cMyc , cDazl,* and *Cvh* genes.

The transmission rate of chimera was reported to improve by altering the ratio of donor and recipient germ cells by irradiation [19], chemical treatment [20], or physical removal [5]. In our present study, the recipient eggs were irradiated with X-ray, which reduced up to 94.8% of endogenous germ cells at stage 28 (data not shown). In this study, X-ray irradiation significantly improved the transmission rate from 54.1% to 81.7% in the group with cell line 1110 g.64. However, irradiation did not significantly improve the transmission rates of the chimeras from the cell line 1110 h.79. This may be possibly caused by a different batch of irradiation.

Many reports have indicated that the sex differentiation of PGCs is directed by the external signals in the gonadal environment, and not by the germ cells themselves [21]. Differentiation of chicken ZZ PGCs in the opposite-sex female chimera was reported by transferring circulating PGCs, gonadal PGCs, and blastoderm cells. The germline transmission rate was only 0.4%–0.9% in the chimera (ZZ/ZW) by transferring circulating PGCs [6] and gonadal PGCs [8]. The extremely low transmission rate made the result inconclusive. In this study, when transferring large number of pure ZZ PGCs into irradiated recipient male embryos, the frequency of germline transmission was 34.1 times higher than that in female chimeras (81.9% vs 2.4%). Particularly, in the bird x126, ZZ PGCs derived functional ova contributed 27.8% (5/18) of the offspring. These results suggest that increasing the ratio of exogenous germ cells by transferring higher number of in vitro cultured PGCs and deletion of endogenous PGCs can also improve the chances of ZZ PGCs in functional ova production.

The plasticity of sex differentiation in germ cell has also been reported in other species. For instance, germ cells of rainbow trout at various developmental stages, e.g., PGCs, spermatogonia stem cells, and premeiotic oogonia, were capable to colonize in sexually undifferentiated embryonic gonad of the same or opposite sex, and further gave rise to both functional spermatozoa and eggs [4, 22]. Chicken testicular and ovarian gonocytes from 20-day-old embryos were still capable to migrate, and contribute to germline after transfer into recipient embryos at stage 14 [23]. It is likely that the plasticity of sex differentiation of germ cell remained until spermatogonia stem cells in male and premeiotic oogonia in female. However, it is not clear whether these developmentally advanced germ cells will adapt to the pace with endogenous germ cells or maintain the existing developmental process at their own pace. The germline transmission of opposite-sex chimera confirmed the possibility of functional ova differentiation from chicken ZZ PGCs. However, the efficiency of ZZ PGCs differentiation into ova is still far lower than that into spermatozoa in nature. The generation of functional ova from PGCs is a long process involving migration, colonization, mitotic proliferation, differentiation, and meiotic stages. When transferring equal number of chicken gonad PGCs, Nakajima et al. reported that the number of donor PGCs did not show significant difference in the gonad of recipient embryos among any of the four possible same and opposite-sex combinations [8]. In the model of sex reversed male chicken (ZW), Abinawanto et al. [25] found that the ZW germ cells could complete the second meiosis, and the transformation of spermatid to spermatozoa was partially impaired. It is still unknown how and at what stage of oogenesis ZZ PGCs differ from their counterpart the endogenous ZW PGCs, either during mitotic proliferation or the process of meiosis in the ovary of the host.

What made us curious was to cross the female (ZZ/ZW) and male (ZZ/ZZ) chimeras of the same donor PGCs origin. Compared to conventional inbreeding program, the uniparental offspring were expected to preserve or restore most of the genetic information of a specific donor rooster. In this study, genotyping results showed that the homozygosity of the 13 loci from the detected six offsprings varied from 61.5% to 84.6% (Table 5), which increased 30%–40% than that of the donor birds (38.5%) in one generation. In addition, the comparison of genotype profile in the 13 loci showed that the uniparental offspring was 100% derived donor PGCs. In the present method, the donor rooster transmitted 69.2%–88.5% genetic information to his uniparental offspring, instead of 50% in conventional breeding. Breeding using opposite-sex chimera will enhance the chances to achieve higher homozygosity in a shorter period than a conventional inbreeding program, and provide a new approach to generate specific chicken line with interesting traits in a short time period. The complete oogenesis of ZZ PGCs may reveal that W chromosome is unessential for ova development. Additionally, in sexual reproduction or somatic cell nuclear transfer-derived animals, the mitochondrial DNA is maternally inherited. Although the mitochondrial DNA information of these uniparental chicken clones was not analyzed, it likely originated from the donor PGCs. Therefore, uniparental chickens may provide a desired model for gametogenesis research, and novel approaches for conservation and restoration of the avian genetic resources.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Figure S1. Genotype analysis of uniparental chick x126-6, PGC line 1110 g.64, x104(ZZ/ZZ), and x126 (ZZ/ZW) with MCW295, HUJ2, and MCW183 markers.
Supplemental Table S1. Progeny test of homosexual chimera (ZZ/ZZ) of PGC line 1110 h.79.
Supplemental Table S2. Progeny test of heterosexual chimeric females (ZZ/ZW) with cell line 1110 h.79.
Supplemental Table S3. List of primers and sequences.
Supplemental Table S4. Primary antibodies information.

Acknowledgments

The authors are grateful to Dr. Noce, Keio University, Japan, Dr. Ali Ridha, the administrative director of central veterinary research laboratory (CVRL), Dubai, UAE, Dr. Tony Bill, Al Tawam hospital, Al Ain, UAE, for their technical supports.

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